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Biological nitrification inhibition by rice root exudates in two different soils of Uruguay

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Abstract

Rice root exudates can exert control over soil nitrification by releasing inhibitory compounds denominated Biological Nitrification Inhibitors (BNIs). Few studies demonstrated how this BNI capacity responds to different soil types and how it affects nitrification populations.

We aimed to know if the root exudates of the main high yielding rice cultivars sown in Uruguay had the ability of BNI in two soils with different organic matter (OM) content and if they decrease the abundance of ammonia-oxidizing bacteria (AOB) or archaea (AOA). We used the bioluminescence assay to evaluate BNI of root exudates at different rice growth stages and a soil microcosm experiment to measure root-exudate-induced nitrification inhibition.

We found that exudates from both indica and japonica genotypes possess BNI potential and a different BNI capacity among them. They also differ in soil nitrification inhibition depending on the soil type.

No evident effect was detected in AOB or AOA populations after 12 days of soil incubation. Both soils presented similar patterns of AOB abundance per treatment application independently of their different copy numbers of bacterial amoA genes. AOA populations were more abundant than AOB for all treatments and types of soil. However, only a significant relationship was found between soil nitrate (NO3-) content and the number of AOB in both soils.

These findings improve the understanding of the BNI capacity of rice and the interaction of its expression with soil OM content.

Keywords: ammonia oxidizing activity, AOA, AOB, bioluminescence assay, microcosm incubation

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Authors´ contributions

Conception of the work and funding request were done by GI and PI. Material preparation, data collection and analysis was performed by GI with supervision of PI. Part of the work was done at CIAT in JA laboratory with help of JN. The first draft of the manuscript was written by PI and GI and all the authors commented on previous versions. All authors reviewed, edited and approved the final manuscript.

Data availability

The datasets generated during the current study are available on reasonable request

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval Not applicable.

Consent to participate Not applicable.

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Introduction

It is estimated a world nitrogen (N) fertilizer demand of around 110.000 thousand tonnes N for 2020 (FAO 2017). Industrial N fixation to produce ammonium (NH_4^+) fertilizers accounts for approximately two percent of the world's energy consumption (Wendeborn 2019). But crops use fertilizer N inefficiently as more than 50 % of its N is not assimilated by plants. Particularly, N-use efficiency in rice is around 44% (Ladha et al. 2005).

The microbial process of nitrification is the oxidation of ammonia (NH_3) to nitrite (NO_2^-) and NO_3^- and this is the major regulator of the loss of soil N from terrestrial ecosystems (Banning et al. 2015), raising production costs and contributing to water pollution and climate change (Canfield 2010). NO_3^- can be lost by runoff, leaching and denitrification, producing environmental problems such as groundwater contamination, eutrophication of freshwater or losses as nitrous oxide (N_2O), a greenhouse gas with 300 times the heat-trapping capacity of carbon dioxide (CO_2) and that also produces ozone and air quality degradation (Di and Cameron 2002; Erisman et al. 2013).

Autotrophic nitrification, the most relevant in soils, occurs in aerobic conditions as a two-step process, wherein the initial oxidation of NH_3 to hydroxylamine is catalyzed by the enzyme ammonia monooxygenase (AMO) present in AOB and AOA (Lan et al. 2018). The *amo*A gene, encoding a subunit of AMO, is a functional marker used to identify AOB and AOA (McCarty 1999). Archeal nitrifiers may be more abundant in paddy soil than bacterial nitrifiers (Chen et al. 2011; Wang et al. 2014), even though in paddy soils from Uruguay AOB predominated over AOA (Azziz et al. 2016). Nevertheless, abundance does not mean functional predominance and while Wang et al. (2011) have found that nitrifying potential depends on AOB abundance, others authors found no correlation of AOA or AOB abundances with nitrifying potential (Boyle-yarwood et al. 2008).

Attempts to control nitrification in agricultural soils, blocking NH_3 oxidation, have recently become the focus of many researches (Coskun et al. 2017) by deactivating AMO. Numerous compounds including nitrapyrin (Parkin and Hatfield 2010), dicyandiamide (DCD) (Di et al. 2009a), and 3,4-dimethylpyrazole phosphate (DMPP) (Zerulla et al. 2001) have been identified to slow down nitrification in agricultural systems. Although the high

cost of these compounds, the variable effectiveness due to rapid degradation rates in soils or difficulties with application, among others (Fillery 2007), have limited their adoption. In general, nitrification inhibitors suppressed AOB growth (Di et al. 2009a; Gong et al. 2013; Meng et al. 2020), but there are some reports that transcripts of the bacterial *amo*A gene but not the *amo*A gene copy number were affected (Florio et al. 2014).

Recent researches suggest that plant roots can release nitrification inhibitory compounds denominated BNIs (Subbarao et al. 2006, 2009). These findings offer possibilities for using BNI as a low-cost *in situ* biological alternative to synthetic nitrification inhibitors. The BNI potential of root exudates has been initially estimated by a bioluminescence assay using a recombinant *Nitrosomonas europaea* strain (harbouring *lux*AB genes from *Vibrio harveyi*) (Subbarao et al. 2006). This methodology was applied for the tropical grass *Brachiaria humidicola* (*Bh*) which has the highest reported BNI potential (Lu et al. 2019) and its BNI compound was identified as brachialactone (Subbarao et al. 2009).

Field crops have been evaluated for BNI capacity and whereas legumes seem to stimulate nitrification (Subbarao et al. 2007a), cereals showed variable behaviours. Several BNIs exuded from roots have been identified, such as sorgoleone and methyl 3-(4-hydroxyphenyl) propionate (MHPP) in sorghum (Subbarao et al. 2013a) but BNI in staple foods as rice, wheat, and maize has been less investigated (Coskun et al. 2017). Pariasca Tanaka et al. (2010) showed that only some rice primitive genotypes root exudates possessed BNI ability. However, Sun et al. (2016) confirmed BNI potential in both *indica* and *japonica* rice genotypes, and identified 1,9-decanediol as their BNI compound. Once checked enough BNI activity on *Nitrosomona*s sp. in pure cultures, it is necessary to test the effectiveness in the field (Wendeborn 2019). Lu et al. (2019) found that 1,9-decanediol decreased AOB and AOA abundance in three different soils and changed AOB community structure.

The ability of BNI not only depends on the plant variety but also on the growth stage of the plant that determines root exudation and on the soil type (Gopalakrishnan et al. 2009). However, the effects of soil type and its OM content have been scarcely investigated (Ipinmoroti et al. 2008; Subbarao et al. 2012).

Rice (*Oryza sativa* L.) is a staple food that supplies essential calories for approximately a quarter of the world's population and is one of the main products of Uruguayan agriculturally based economy. Rice is associated with environmental concerns and one of them is the increased N-fertilizer application for booting yields. Agronomic management changes have increased Uruguayan rice yield, using locally developed rice cultivars, to around 8 Mg ha^{-1}, the 3rd highest in the world (Tseng et al. 2020).

The aim of this study was to determine if the root exudates of the main rice cultivars sown in Uruguay, one *indica* and one *tropical japonica*, had the ability of BNI in different rice paddy soils with different OM content and decreased the abundance of AOB or AOA. We used the bioluminescence assay and a soil microcosm experiment to evaluate BNI of root exudates of different rice growth stages over potential soil nitrification activity and abundance of AOB and AOA.

We therefore hypothesized that both high yielding rice cultivars possess different BNI activity, that this activity differs with the soil type and is linked to AOB population.

Materials & Methods

Soil samples

The soils used in this study were collected from the top 15 cm of two paddy fields two months before rice sowing. The two sites selected represent the two main geographical zones where rice is cultivated in Uruguay. One soil was collected at Paso de la Laguna Experimental Station of the Instituto Nacional de Investigación Agropecuaria (INIA) (33°16¨10" S; 54°10' 04" W), and the other soil was collected from a commercial paddy field (31°22'10" S; 57°27' 45" W). The two soils will be referred hereafter as Treinta y tres, and Salto, respectively. Treinta y tres was a Typic Argiudoll, with pH 5.7, 3.4% OM, 13 mg/g P Bray I, and 10 mg/g NO_3-N. Salto soil was a Typic Hapludert, with pH 5.7, 5.8% OM, 10 mg/g P Bray I and 15 mg/g NO_3-N. Treinta y Tres soil had significantly lower OM content than Salto soil. The stones, roots and debris were removed from the soils which were partially dried for a day before being passed through a 2 mm-sieve. A subsample of the sieved soils was used to determine the moisture content by drying at 105°C for 24 h. Other soil subsamples were used to determine the soil water holding capacity.

Cultivation of rice plants and collection of root exudates

Seeds of the two rice cultivars, El Paso 144 (*O. sativa* ssp. *indica*) and Tacuarí (*O. sativa* spp. *japonica*), hereafter El Paso and Tacuarí, were surface sterilized and pre-germinated in water-agar. Three germinated seeds were sown in each plastic bag (n=4) corresponding to a cultivar and a soil type. The plastic bag (4 kg capacity) contained a mixture of each soil and washed and sterilized sand (1:1 ratio) and were fertilized with $(NH_4)_3PO_4$

(0.49 g per plastic bag). The plants were grown in a greenhouse for 56 days and 60% water holding capacity was maintained with distilled water. The plants were harvested at 32 and 56 days after sowing. According to other studies that obtained root exudates from plants growing on soil (Canarini et al. 2016; de Vries et al. 2019), manipulation of roots were carefully controlled in order to remove the soil without injuring the roots. Following the procedure of Aulakh et al. (2001), the plastic bags were cut open and the soil around the roots was washed off with gentle water spray and last washing was given with distilled water. The root system of each replicate was rinsed consecutively with distilled water and trap solution (1 mM NH_4Cl and 200 µM $CaCl_2$) and transferred to jars, where their roots were immersed in a 500 mL dark bottle containing aerated trap solution for 24 h to collect root exudates (Pariasca Tanaka et al. 2010). After 24 h, the roots were saved for dry matter determination. The trap solution was evaporated to dryness using a rotary evaporator (Buchi, R-114, Switzerland) at 40°C, then re-suspended in 5 mL 100 % methanol, passed through a syringe-driven 0,22 µm membrane filter (Ministat, Millipore, USA) and re-evaporated twice. Finally, the concentrated sample was suspended in 25 µL of dimethyl sulfoxide (DMSO). These rice root exudates were used for the bioluminescence assay.

To test the effect of rice root exudates in soils, larger quantities of exudates were necessary. To obtain the exudates for the microcosm assay, 20 rice seedlings were hydroponically grown in each plastic box (40 x 60 x 32 cm) for a month at 25°C and a photoperiod of 16:8 h, light:dark with aerated Yoshida mineral solution (Subbarao et al. 2006). There were 3 replicates (boxes) for each rice cultivar whose roots were submerged in the trap solution for 24 h. The solution was filtered (0.22 µm pore) and lyophilized. The samples were resuspended in 10 ml methanol and rotoevaporated twice and finally resuspended in 1.5 ml of distilled water (Pariasca Tanaka et al. 2010). The same procedure was employed with the trap solution as a control.

Nitrification inhibition potential bioassay

The bioassay was performed with a recombinant *N. europaea* strain that had been transformed with a plasmid carrying the luciferase gene (Iizumi et al. 1998) and standardized for estimation of BNI potential by Subbarao et al. (2006). The strain produces bioluminescence due to the expression of the *lux*AB gene which is reduced when exposed to a nitrification inhibitor. This strain was provided by J. Arango and the assay was conducted at the Laboratory of Tropical Forages Program, in Centro Internacional de Agricultura Tropical (CIAT; Cali, Colombia). The *Nitrosomonas* strain was grown in P-medium (KH_2PO_4 5.14 mM, Na_2HPO_4 95.1 mM, $(NH_4)_2SO_4$ 18.91 mM, $NaHCO_3$ 5.95 mM, $CaCl_2$-$2H_2O$ 0.034 mM, $MgSO_4$-$7H_2O$ 0.041 mM, Fe (III) EDTA 0.0027 mM, pH 7.8), during 7 days at 150 RPM and 28°C in darkness, supplemented with Kanamycin 50 mg ml^{-1}. After that, the culture was centrifuged and the pellet obtained was resuspended in 50 mL P-medium.

A mix of 2 µl of root exudate (suspended in DMSO) with 198 µl of distilled water and 250 µl of bacteria (DO 0.6), was incubated for 15 min at 15°C with continuous shaking at 700 RPM (Thermomixer eppendorf 3420). Luminescence was measured with a luminometer glomax 20/20 (promega) with injection of 25 µL of n-decil-aldehyde 1% (luciferase substrate). The luminescence was registered as the integration time between 2 and 10 s. Each sample had 4 independent replications. Root exudates from *Bh* CIAT16888 with recognized nitrification inhibition activity (Arango et al. 2014) was used as positive control. The root exudates were obtained from hydroponically grown stolons for 90 days and then trapped in solution with NH_4Cl and $CaCl_2$ as described in Nuñez et al. (2018).

The allylthiourea (ATU) units were calculated considering an inhibition of 80% of luminescence of 0.22 mM of ATU, according to Subbarao et al. (2006).

Microcosm set- up and measurement of potential nitrification inhibition in soils

Soil microcosms were established as Nuñez et al. (2018) with some modifications. They consisted of 25 mL vials containing the equivalent to 5 g of dry homogenized soil whose 60% water holding capacity was maintained with the addition of the different treatments.

The treatments were: (1) Nitrogen, 0.2 g N kg^{-1} soil provided as a $(NH_4)_2SO_4$ solution, (2) ($(NH_4)_2SO_4$) plus 0.5 ml root exudate of Tacuarí, (3) ($(NH_4)_2SO_4$) plus 0.5 ml root exudate of El Paso, (4) ($(NH_4)_2SO_4$) plus 20% DCD, a synthetic nitrification inhibitor and, (5) ($(NH_4)_2SO_4$) plus trap exudates solution. All treatments were prepared to contain the same concentration of N and $(NH_4)_2SO_4$ solution was adjusted according to exudates and DCD N content to create non-limiting conditions to estimate the nitrification potential. To monitor NH_4^+ and NO_3^- dynamics without added N, vials with only soil and distilled water were prepared.

The microcosms were covered with Parafilm® to allow gaseous exchange and kept at 25°C in the dark for 12 days. To determine the nitrification dynamics, NO_3^- content of 3 technical replicates were analyzed at 0, 6 and 12 days taking destructive samples for all the treatments.

Soil suspensions, 4g of soil with 40 mL of 2M KCl shaken 30 minutes at 200 RPM, were filtered through Whatman filter Nº 42. N-NO_3^- content was determined colorimetrically after reduction through a Cd column (Griess-Ilosvay reaction, Mulvaney (1996). N-NH_4^+ was determined colorimetrically (660 nm) according to *Berthelot* method (Rhine et al. 1998).

Nitrification rate in each replicate was calculated as the slope of the increase in N-NO_3^- concentration during the 12 days of incubation. The relative nitrification rate to the Nitrogen treatment was calculated as the Nitrification inhibition (%) for DCD and root exudates.

In the case where trap solution had a nitrification inhibition effect *per se,* nitrification inhibition was compared to that of the trap treatment. This BNI effect of the exudates trap solution had been reported previously (Sun et al. 2016).

Soil DNA extraction and ammonia-oxidizing abundance

The AOA and AOB abundance were estimated through real time quantitative PCR (qPCR) using the *amo*A gene marker on samples of days 0 and 12 of the microcosm assay. Total genomic DNA was isolated from 0.25 g of soil using PowerSoil DNA-isolation kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The extracted DNA was checked on a 0.8% agarose gel. The DNA concentration and purity were determined with NanoDrop® 2000c UV–vis spectrophotometry (USA). The qPCR was done on a *Rotor-Gene* 6000 thermocycler (*Corbett Research Ltd., UK*), using the fluorescent dye SYBR-Green I. All of the samples and standards were quantified in triplicate. The reaction mixture for AOA (12.5 µl) contained 6.25 µl of 2 Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific Inc.), 10 µg of bovine serum albumin, 2.5% (v/v) DMSO and 0.5 µM each primer: Arch-amoAF (5´-STA ATG GTC TGG CTT AGA CG-3´) and Arch-amoAR (5´-GCG GCC ATC CAT CTG TAT GT-3´) (Francis et al. 2005). Thermal cycling was as follows: 95º C for 10 min and 40 x (95º C, 45 s; 53º C, 45 s; 72º C, 45 s; and 79º C, 15 s for data collection), and the program ended with a melt curve from 65º C to 90º C (Azziz et al. 2016). Standard curves were generated by amplifying 10-fold dilutions of a mix of linearized pJET1.2/blunt plasmids containing five different archaeal *amo*A genes. The PCR efficiency ranged from 85% to 102% averaging 95%, and the correlation coefficient was on average 99%.

The reaction mixture for AOB (12.5 µl) contained 6.25 µl of *QuantiNova SYBR Green* (Qiagen), 10 µg of bovine serum albumin, 2.5% (v/v) DMSO, 1 ROX reference dye and 0.4 µM each primer: amoA-1F (5´-GGG GTT TCT ACT GGT GGT-3´) (Rotthauwe and Witzel 1997) and amoA-2Rs (5´-CCT CKG SAA AGC CTT CTT C-3´) modified from those originally published by Rotthauwe and Witzel (1997) to minimize dimmer formation. Thermal cycling was as follows: 95º C for 10 min and 40 x (95º C, 30 s; 58º C, 30 s; 72º C, 45 s; and 80º C, 15 s for data collection), and the program ended with a melt curve from 72º C to 90 ºC. Standard curves were generated by amplifying 10-fold dilutions of linearized pJET1.2/blunt plasmid containing *amo*A gene from *N. europaea*. The PCR efficiency ranged from 85% to 104%, averaging 98%, and the correlation coefficient ranged from 0.91 to 0.99, averaging 0.96.

Gene abundances were standardized by the mass of DNA that was extracted per gram of dry soil and log10 transformed before analysis.

Statistical analyses

The differences between treatments were assessed by analysis of variance (ANOVA) after analyzing the homogeneity of variance and the residuals by Shapiro–Wilk and Levene's tests and verifying interaction among factors. Tukey's HSD *post-hoc* analysis was used to determine significance of soil type and treatment effect on nitrification inhibition. The InfoStat software was used for statistical analysis (Di Renzo et al. 2018). The significance level was $\alpha = 0.05$.

Regressions were performed to understand relationships between soil NO_3^- content and AOB and AOA populations.

Results

BNI potential of rice root exudates

The root exudates of the two rice cultivars grown in the two types of soil presented potential BNI activity (Fig. 1) when using a bioluminescence assay with a recombinant *N. europaea* strain. There were no differences in the BNI activities of root exudates obtained from 32 or 56 days´ after sowing plants for either cultivar or type of soil. The type of soil (Salto and Treinta y tres) did not significantly affect BNI. When considering all the results for the 2 dates and the 2 soil types, rice cultivar Tacuarí presented higher BNI than cultivar El Paso, average 354

and 209 ATU g-1 dry weight of root, respectively (Fig. 1). The BNI activity expressed as inhibition percentage was 45 ± 9 % for Tacuarí and 38 ± 12% for El Paso, respectively. Both rice root exudates resulted in less BNI activity than *Bh* CIAT 16888, 491 ATU g^{-1} root (Fig. 1).

Nitrification inhibition in two different soils

Nitrification rates of the two soils for the different treatments plus $(NH_4)_2SO_4$ amendment are shown in Fig. 2. The rate of increase in $N\text{-}NO_3^-$ concentration varied within the 12 days of soil incubation between Salto (higher OM) and Treinta y Tres (lower OM) soils. The mean nitrification rate with only $(NH_4)_2SO_4$ incubation (Nitrogen treatment) was 0,709 and 0,623 mg $N\text{-}NO_3^-$ kg^{-1} soil dry weight day^{-1} for Treinta y Tres and Salto soils respectively. The addition of root exudates from both rice cultivars, El Paso and Tacuarí, and also DCD, significantly decreased nitrification rate for Salto and Treinta y tres soils. Although not significant, mean nitrification rates for Tacuarí were lower than for El Paso in both soils. The nitrification rate of N plus the trap solution used to collect the exudates was lower than that of N for the Salto soil (0,119 mg $N\text{-}NO_3^-$ kg^{-1} soil dry weight day^{-1}), meaning an unspecific nitrification inhibition of the trap solution.

The nitrification inhibition percentage of root exudates and DCD for the two soils is presented in Fig. 3. The percentage decrease in nitrification rate within 12 days incubation was similar for the two rice cultivars and the synthetic nitrification inhibitor DCD (ranging from 50 to 96%) for Treinta y Tres soil. For Salto, the soil with higher OM content, the range of inhibition of exudates from both rice cultivars and DCD was between 60 and 96%. But, considering the unspecific nitrification inhibition in trap solution for Salto soil, root exudates from cultivar Tacuarí inhibited nitrification 32% while cv. El Paso did not produce nitrification inhibition.

Effect of root exudates on AOA and AOB abundance

The total bacteria *amo*A gene copy number in Salto at the beginning ranged from 1.2 (± 0.1) to 2.1 (± 1.1) x 10^6, whereas in Treinta y Tres from 3.7 (± 0.9) to 6.5 (±2.3) x 10^5 g^{-1} dry soil weight (Fig. 4). So, Salto soil, with higher OM content, had a larger AOB population size than Treinta y tres (p= 0.014).

The AOB population abundance after 12 days with Nitrogen treatment did not change in any of the soils assayed. Applying DCD or root exudates of both rice cultivars did not inhibit AOB growth significantly, although the average *amo*A copy numbers were lower than for N (Fig. 4) in both soil types.

Archeael *amo*A gene abundance was an order of magnitude higher compared to bacterial *amo*A with a mean of 1.5 x 10^7 for Salto and 1.1 x 10^6 g^{-1} dry soil weight for Treinta y Tres (Fig. 4). The *amo*A archeal genes copy number fluctuated less than their bacterial counterpart in this experiment. As for bacteria, AOA population was higher in Salto soil than in Treinta y Tres ($p < 0.0001$). The copy number of archeae *amo*A increased twice after 12 days of incubation. There was no significant effect in AOA population abundance among treatments in any of the soils (Fig. 4).

A significant linear correlation was observed between soil $N\text{-}NO_3^-$ concentration and AOB abundance while not with AOA (Fig. 5).

Discussion

Root exudates of Tacuarí and El Paso rice cultivars inhibit nitrification

The ability to decrease soil nitrification by the release of nitrification inhibitors by plant roots has been well documented (Subbarao et al. 2013b; Coskun et al. 2017). Tropical grasses, like *Bh*, adapted to low N-environments presented high BNI (Subbarao et al. 2006). It is crucial to survey cultivars of major crop plants for this activity (Norton and Ouyang 2019). In this work we evaluated the two most planted rice cultivars in Uruguay, where this grain is one of the main export products.

The characterization of the BNI trait has been facilitated by the development of a bioassay using a luminescent *N. europaea* to quantify nitrification inhibition by root exudates. Using this bioassay, the two rice cultivars evaluated presented specific BNI activity although lower than that of the used control pasture grass *Bh* CIAT1688 (Fig. 1). Initial screening studies had not found BNI activity in rice cultivars (Subbarao et al. 2007a), whereas other studies concluded that traditional and/or upland varieties presented higher BNI than high-yielding lowland varieties (Pariasca Tanaka et al. 2010). The high yielding cultivars INIA Tacuarí and El Paso 144 evaluated in this work, are planted in dry soil but flooded since tillering to harvest. Their registered BNI activity was in the range of the highest BNI observed by Pariasca Tanaka et al. (2010) for other rice varieties. Furthermore, Sun et al. (2016), without using the luminescent bioassay, found that root exudates from both *indica* and *japonica* genotypes possessed BNI potential. In our study, Tacuarí ssp. *japonica* had more BNI potential than El Paso ssp. *indica*. BNI did not vary with the plant stages of growth tested, 32 and 56 days after

sowing. However, other authors found that the inhibition of nitrification increased (Zakir et al. 2008; Sun et al. 2016) or decreased with plant maturity (Pariasca Tanaka et al. 2010; Subbarao et al. 2013a). These different results might be caused by the different varieties used and different growth and exudates collection conditions (Sun et al. 2016).

In our study we choose the soil-hydroponic-hybrid approach to more realistically estimate root exudation under natural soil growth conditions compared to artificial hydroponic systems (Oburger and Jones 2018). Collection of root exudates from soils may better represent their hydrophilic and hydrophobic composition and (Nardi et al. 2020) encourage this approach to better understand BNI effect. Subbarao et al. (2012) proposed that it is likely that BNI-function is better expressed in plants when grown on light-textured soils with a pH 6.0 or lower, but have not yet been evaluated using soil systems. The two different soils compared in this work, with contrasting OM content, didn`t influence rice root exudates BNI ability when evaluated with the bioassay.

Soil nitrification rates determined through microcosm incubation represent a complementary methodology to the in vitro bioassay, for assessing the expression of BNI potential in soil and evaluate the behavior or rice exudates in front of other ammonia-oxidizers apart from a sole *N. europaea* strain.

The nitrification rate in each replicate was estimated from the dynamics of $N\text{-}NO_3^-$ concentration during soil incubation. Using the rate of change of $N\text{-}NH_4^+$ instead, could have led to different conclusions since it can react with OM, be fixed in clay particles or be immobilized in microorganisms. We found that nitrification rates varied with the soil type. The two soil types evaluated in this work presented similar texture although Salto has almost twice OM content. Soil OM might reduce the effectiveness of nitrification inhibitors as occurred in this work, either by stimulating degradation microbial activity or by reducing the bioactivity of inhibitors through absorption on the soil colloids (Rajendran 2011). However, for both soil types, the addition of rice exudates from both cultivars evaluated significantly slowed down nitrification (Fig. 2). In the Salto soil, the trap solution used for collecting rice root exudates had a similar nitrification rate than the exudates themselves. This nonspecific inhibition effect has been previously reported by Pariasca Tanaka et al. (2010). These authors attributed the effect to the high electric conductivity of the trap solution due the remnant NH_4Cl. Sun et al. (2016) proposed to use only water to collect the exudates to avoid this effect and other authors to use a column to remove inorganic salts (Gopalakrishnan et al. 2009, Li et al. 2021).

The high inhibition of nitrification registered for both rice cultivars exudates (Fig. 3) in Treinta y Tres soil respect to the sole N treatment, was not an indirect effect of N net immobilization as there was not a decline in NH_4^+ supply during the 12 days evaluated (data no showed) and due to the high NH_4^+ concentration applied to the microcosms. On the other hand, Vázquez et al. (2020) reported under low N environment for *Bh* that BNI effect was not only the result of gross nitrification suppression but mainly the product of a higher inorganic N immobilization by microbes. In Salto soil only Tacuarí cultivar showed BNI activity in comparison to the trap solution. This cultivar also had the highest BNI potential determined by the luminescence bioassay. The soil physico-chemical and biological properties of the Salto soil could have interfered with BNI molecules as has been reported with a sorghum exudate organic compound (Subbarao et al. 2013a). Ipinmoroti et al. (2008) suggested that in soils with high OM content, like the Salto one, the organic compounds soil matrix could interfere with BNI as happened in an Andosol with exudates of *Bh*.

Some of our results need further discussion as the BNI effect measured for 12 days may be transitory as has been reported by different authors (Subbarao et al. 2006; Pariasca Tanaka et al. 2010). On the other hand, root exudates of a wild relative of wheat, *Leymus racemosus* (Lam.), suppressed nitrification during more than 60 days (Subbarao et al. 2007b). Even more, Moreta et al. (2014) and Karwat et al. (2017) found that the BNI effect of Bh on soil nitrifier activity could persist for at last one year after replacement of the pasture with maize. It would be necessary to characterize the inhibitory compounds exudated by these high -yielding rice cultivars and their mechanism of action as well. 1,9 -decanediol has been identified as a nitrification inhibitor produced by rice roots (Sun et al. 2016) that blocks AMO. Its exudation has been correlated to plant NH_4^+ preferences, a feature that must be tested for the rice cultivars assayed that had different BNI activity, at least in one of the soils. But both rice cultivars are adapted to NH_4^+ nutrition at least during their flooded period where NO_3^- could not be detected (Irisarri et al. 2007).

In order to consider BNI as a valid alternative to regulate nitrification in rice paddy soil it must be taken into account the high concentration of exudates and N used in the microcosms assay. Souri and Neumann (2017) claimed that in the case of *Bh* the release of BNI substances from root exudates may not be an active process but a passive phenomenon induced by NH_4^+ used to collect them during a long period (24 h) that may have produced membrane damage. Furthermore, O'Sullivan et al. (2016) demonstrated that under hydroponic conditions not all the BNI potential observed in the root tissue is exuded. On the other hand, root tissue extracts from *Bh* showed BNI potential (Nuñez et al. 2018), rejecting the fact that exudates collection methodology introduced an

experimental artifact and did not actually produce nitrification inhibition. Besides, brachilactone was identified in root exudates from *Bh* collected either with NH_4Cl or with water by chromatography and mass spectrometry (Arango et al. 2014). Yang et al. (2017) showed that for rice N metabolism, signaling and defense/stress response are interconnected and this fact may be taken into account when considering what will happen in the field.

Similar to other plant species, rice can absorb both NO_3^- and NH_4^+ mineral forms of N (Poletto et al. 2011). The same author reported that upland rice performs best when NH_4^+ is predominant during the initial development of plants and is also the preferential form of N applied as fertilizer. The BNI effect during the first period after basal fertilizer application will be desirable, especially if rice is sown in dry soil, and so this trait deserves further experimental research.

The nitrification inhibitor DCD was used as a positive control of NI. DCD is an inhibitor of AMO enzyme widely used to reduce nitrification rates in soils and so decrease N losses and improve efficient N use by crops (Liu et al. 2014; Meng et al. 2020). Inhibition of nitrification rates around 99% have been reported in soil incubation experiments (Gopalakrishnan et al. 2009; Zhang et al. 2012). Nitrification inhibition by DCD was around 50-60% for the two soils, lower than the inhibition produced by root exudates except for Salto soil and El Paso cultivar. The sorption phenomenon of DCD by soil OM has been reported as a critical factor for its efficacy (Kelliher et al. 2008).

The rice root exudates did not impair AOB and AOA abundance after 12 days of soil incubation

Previous studies showed that root exudates BNI activity decreased nitrification rates and ammonia-oxidizers populations simultaneously without affecting other soil microorganisms (Gopalakrishnan et al. 2009; Subbarao et al. 2009). In this work no change in AOB populations could be detected after 12 days of soil incubation. Salto and Treinta y Tres soils presented similar patterns independently of their different copy numbers of bacterial *amo*A genes.

Our results indicate that AOA populations were more abundant than AOB for all treatments and types of soil (Fig. 4). Similar results were reported for other paddy soils (Chen et al. 2011; Jiang et al. 2015), while some others found opposite trends (Wu et al. 2011). Previous findings suggested that soil pH, acid in this case, is a key factor driving the niche partitioning of AOA and AOB (Hu et al. 2014; Liu et al. 2015; Shi et al. 2016). The heterogeneity of paddy soils can be responsible for these findings. And in general acid soils support autotrophic nitrification, dominated by nitrifying archaea (De Boer and Kowalchuk 2001; He et al. 2012).

The copy number of bacterial *amo*A genes was in the order of the reported by Azziz et al. (2016) for nearby soils. The soil with higher OM content had around ten times more AOB abundance. Similar results were registered elsewhere (Gong et al. 2013).

Although no change in AOB populations could be detected after 12 days of N addition, the low growth rate of these microorganisms could explain their abundance was not modified in this period. Glaser et al. (2010) found that *amo*A copy number did not change until 21 days after NH_4^+ increase and so 12 days of incubation would have been enough to show nitrification inhibition effects but not to detect changes in AOB abundance. In other cases, a rapid growth of AOB in response to addition of inorganic **N** had been observed in agricultural soils (Jia and Conrad 2009; Lan et al. 2018). Recently, Li et al. (2021) reported that sorghum root exudates significantly reduce AOB but not AOA. However, a significant relationship was found between soil NO_3^- content and the number of AOB obtained from the qPCR technique (Fig. 5). We did not reactivate soil for the microcosms in order to provide 60% field capacity with the solution added, $(NH_4)_2SO_4$ or this solution and exudates or DCD, and so, soil microorganisms may have needed some time for adaptation and 12 days of incubation was not enough to show detectable changes in their numbers. Furthermore, AOB abundance may not be affected but their activity could be. However, there are several reports of significant relationships between AOB abundance and the rate of nitrification (Di et al. 2009b).

In the case of AOA populations, they were also more abundant in Salto soil but they did not correlate with NO_3^- content (Fig. 5). Their abundance increased after 12 days of incubation in all treatments (Fig. 4). Hu et al. (2014) reported an important response of AOA to water, greater than to N-fertilizer. On the other hand, Bello et al. (2019) found that AOA were very sensitive to water stress (matric or osmotic potential) which would explain its growth after water addition with the different treatments.

The copy numbers of archaeal *amo*A genes also fluctuated less within the replicates than their bacterial counterparts in this experiment. This would reveal more differences in the population size of AOB but not in AOA. It has been proposed that AOA would be more stable and less responsive to soil environmental changes than AOB (Francis et al. 2005; Hai et al. 2009).

Population size of both ammonia-oxidizers did not decrease after 12 days of addition of DCD as has been shown in other works although with different lapses of exposition to N fertilizers (Gong et al. 2013). O'Callaghan et al. (2010) and Li et al. (2021) did not observe metabolic response of AOA to DCD addition either. Although with these results we cannot discard AOA responded to NH_4^+ addition increasing their population, but this fact was not correlated with nitrification activity or they were not inhibited by DCD or exudates.

In our study AOB, but not AOA, significantly correlated with NO_3^- soil content (Fig. 5). Lan et al. (2018) suggested that the different compositions of the cell membranes of AOB and AOA may influence the cell membrane permeability of nitrification inhibitors.

In conclusion, these rice high-yielding cultivars showed high BNI activity and potential nitrification inhibition in soil. This work suggests a differential genotype capacity of BNI for the rice cultivars evaluated and significant reduction in soil nitrification activity that differ in behavior in different soils.

To explore BNI ability in rice, which has a C3 photosynthetic pathway, may lead to design strategies to improve N use efficiency by this crop and mitigate environmental concerns.

Figure Captions

Fig.1 Biological nitrification inhibition (BNI) potential in the root exudates of rice cultivars Tacuarí and El Paso cultivated in soils from Salto and Treinta y Tres at 32 and 56 days after sowing, and overall BNI potential of the 2 dates for each rice cultivar compared to that produced by *Brachiaria humidicola* CIAT 16888. Data are shown as means (n=4). Error bars represent standard error of the means. Different letters indicate significant differences among treatments ($p \leq 0,05$).

Fig.2 Average net (0-12 days) nitrification rate from Salto and Treinta y Tres soils treated with: exudates of rice cultivar Tacuarí, exudates of rice cultivar of El Paso, exudates´trap solution, the synthetic inhibitor dicyandiamide (DCD) and only Nitrogen supplied as $(NH_4)_2SO_4$. Data are shown as means (n=3). Error bars represent standard error of the means. Different letters indicate significant differences among treatments ($p \leq 0,05$) for each soil.

Fig.3 Nitrification inhibition produced during 12 days of soil incubation amended with: root exudates of rice cultivar Tacuarí, root exudates of rice cultivar El Paso or synthetic inhibitor dicyandiamide (DCD) in Treinta y Tres and Salto soils. Data are shown as means (n=3). Error bars represent standard error of the means. Different letters indicate significant differences among treatments ($p \leq 0,05$) for each soil.

Fig.4 Abundance of archaeal *amo*A genes (a) and bacterial *amo*A genes (b) in soils from Treinta y Tres and Salto at 0 and 12 days of incubation with root exudates of rice cultivar Tacuarí, root exudates of rice cultivar El Paso, synthetic inhibitor dicyandiamide (DCD) and only Nitrogen supplied as $(NH_4)_2SO_4$. Error bars represent standard error of the mean (n=3).

Fig.5 Relationship between soil nitrate content and ammonia oxidizing, archaea (AOA) and bacteria (AOB) populations, for Salto and Treinta y Tres soils. a. AOA in Salto soil, b. AOA in Treinta y tres soil, c. AOB in Salto soil, d. AOB in Treinta y Tres soil. Soil $N\text{-}NO_3^-$ had only a significant positive linear correlation with bacterial *amo*A gene copy number ($p \leq 0,05$).

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